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2. Patent application number *(The Patent Office will fill in this part)* — **9901499.5**

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

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GB

Patents ADP number *(if you know it)* 6200541001

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention — BIOLOGICAL CONTROL

5. Name of your agent *(if you have one)* — MEWBURN ELLIS

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

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Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

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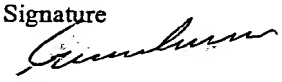
Signature  Date 21 January 1999

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BIOLOGICAL CONTROL

TECHNICAL FIELD

The present invention relates to methods and materials for controlling nematodes, and in particular such methods and materials which are derived from bacteria.

PRIOR ART

Several thousand species of nematodes (sometimes called eel worms) are known, and numerous of these attack and parasitize humans and animals in which they cause disease. Additionally, several hundred species are known which are known to feed on living plants. Certain of these are reviewed by Agrios in "Plant Pathology - 3rd Ed" Pub Academic Press Inc (see Chapter 15 therein).

Methods of controlling nematodes and their associated diseases include cultural practices, biological methods (e.g. use of resistant varieties), physical methods (e.g. heat) and use of chemical agents.

Patent application WO 92/19739 (Mycogen) relates to genes and gene fragments from *Bacillus thuringiensis* which have nematocidal activity. These generally encode crystal toxins from particular strains.

Patent application EP 0 303 426 (Mycogen) also relates to strains of *B thuringiensis* which have nematocidal activity.

Patent application EP 0 171 381 (Monsanto) relates to

particular soil bacteria which are capable of proliferating in an environment which is infested with nematodes (such as pseudomonads which colonise the surface of plant roots). The basis for the controlling activity appears to stem from glycosidase enzymes which are hypothesised to directly inhibit the nematodes.

Notwithstanding these disclosures, there is an ongoing requirement for methods and materials which have nematocidal activity, for instance for use in crop protection or nematode-mediated disease control. Thus it can be seen that such novel methods and materials would provide a contribution to the art.

DISCLOSURE OF THE INVENTION

The present inventors have established that species of bacteria which in nature are associated symbiotically with entomopathogenic nematodes, can in fact be utilised to control nematodes, and in preferred forms of the invention, to kill them.

Although it was known that certain of these bacterial species, such as *Xenorhabdus* and *Photorhabdus*, could be used to control insects (see e.g. WO 98/08388 of MAFF or WO97/17432 of WARF), a demonstrated effect against nematodes had not previously been shown.

The symbiotic bacteria used in the present invention are isolatable from nematodes or the insects which the nematodes attack, and differ fundamentally in terms of life-style and activity from those soil bacteria (e.g. *B. thuringiensis* or

pseudomonads) which have previously been suggested as being nematocidal.

Indeed, *prima facie*, it would seem highly unlikely that nematode symbiotes would possess nematocidal activity. However, in the light of the present disclosure, a number of possible explanations for the observed activity can be tentatively proposed. Firstly, to protect a dead insect nutrient supply the bacteria may produce anti-nematocides to prevent saprophytic nematodes gaining access. Alternatively, to become a symbiont, the bacterial strains may have once been pathogens of these nematodes and evolved towards a less hostile symbiotic relationship. The nematocidal activity may be an evolutionary throwback from the original pathogenic relationship, in which case it may be expected to be widely present amongst bacteria which have evolved in this way.

Thus in first aspect of the present invention there is disclosed use of a bacterial strain to control a target nematode, characterised in that in nature the bacterial strain is associated symbiotically with an entomopathogenic nematode.

As discussed in more detail below, the bacterial strains may be used in the methods of the present invention *per se*, or they may be used as a source of nematocidal agent (which can be derived directly, or be prepared and utilised through recombinant DNA techniques, optionally via a host cell).

The target nematode will generally be different to that with which the bacterial strain is found in nature.

'Control' in this context means to prevent or retard the effect that the nematode has on other organisms such as animals or more preferably plants, or which reduces the number of nematodes or nematode eggs in an area of interest, or which alleviates or cures a disease caused by nematodes. Control may be at the level of larval nematodes or nematode eggs, or may inhibit the motion, feeding or infectivity of adult nematodes. Control may kill the nematode target. Such controlling activity can be assessed as shown in the Examples below.

Some preferred embodiments of the invention will now be discussed.

Bacterial strains

These can be derived from any entomopathogenic nematode. Preferred species are *Xenorhabdus* and *Photorhabdus*.

Potential sources of bacteria for use in the methods of the present invention may be identified by any preferred method. For instance, entomopathogenic nematodes can be isolated using an insect baiting technique such as that described by Bedding & Akhurst (1975) Nematologia 21: 215-227. Bacteria from nematodes identified as being pathogenic to the insect are isolated, cultured, and used as a source of nematocidal agent (e.g. by analogy with the methods used in the Examples below). Preferably *Xenorhabdus* or *Photorhabdus* species are used.

Preferably the bacterial strain is one of these species which has the characteristics of strain C42 isolated by the present

inventors. This *Xenorhabdus* strain has the following characteristics: rod shaped; motile; non-bio luminescent; blue on NBTA; produces anti-biotics; resistant to ampicillin; forms circular colonies; has convex morphology; white colour.

This strain was presumptively identified as belonging to the genera *Xenorhabdus* since it was isolated from an insect killed by an entomopathogenic nematode and had the above characteristics. The strain has been deposited at the NCIMB by the applicants under accession number NCIMB 41004 on 20 January 1999.

Target nematodes and diseases

The group of diseases described generally as helminthiasis is due to infection of an animal (including human) host with parasitic worms known as helminths. Helminthiasis is a prevalent and serious economic problem in domesticated animals such as swine, sheep, horses, cattle, goats, dogs, cats and poultry. Among the helminths, the group of worms described as nematodes causes widespread and often times serious infection in various species of animals. The most common genera of nematodes infecting the animals referred to above are *Haemonchus, Trichostrongylus, Ostertagia, Nematodirus, Cooperin, Ascaris, Bunostomum, Oesophagosromuni, Chaberria, Trichuris, Strongylus, Trichonema, Dictyocaulus, Capillaria, Heterakis, Toxocara, Ascaridia, Oxyuris, Ancylostoma, Uncinaria, Toxascaris, Caenorhabditis* and *Parascaris*. Certain of these, such as *Nematodirus, Cooperia,* and *Oesophagostomum,* attack primarily the intestinal tract, while others, such as *Dictyocaulus* are found in the lungs. Still other parasites may be located in other tissues and

organs of the body.

The bacteria and encoded toxins of the invention may be used as nematocides for the control of the nematodes and diseases discussed above. More preferably, however, they are used to control soil and plant parasitic nematodes. Particular crop species which can be protected include tomatoes, potatoes, sugarbeet, barley, soybean, peanut, onion, rye, wheat, corn, banana, raspberry, beans. Decorative and other plants may also be treated e.g. rose.

Target nematodes may be selected from the genera *Aphelenochoides*, *Anguina*, *Bursaphalenchus*, *Criconemella*, *Melodoigyne*, *Ditylenchus*, *Globodera*, *Helicotylenchus*, *Heterodera*, *Pratylenchus*, *Radopholus*, *Rotelynchus*, *Tylenchus*, *Trichodorus*, *Xiphenema*. A further organism (used in certain of the Examples below) is *Caenorhabditis elegans*. Other target organisms and plants are discussed by Agrios in "Plant Pathology - 3rd Ed" Pub Academic Press Inc (see Chapter 15 therein).

As stated above, the target nematode will generally be different to that with which the bacterial strain is found in nature.

Methods of use of bacteria

The bacteria may be used in any appropriate method which brings them into contact with the target nematode, preferably such that they, or their products, are ingested by the target nematode.

In particular, regarding plants, the bacteria may be formulated in a variety of ways so as to enhance stability.

For instance they may be employed in admixture with substrates to protect the cells.

The mixture can be spread over, ploughed into or otherwise mixed with nematode infected (or potentially infected) soil.

Regarding animals, bacteria intended for enteric inoculation can be mixed with carrier material that is suitable for ingestion by the intended animals.

Isolation of agent

Nematocidal agents of the present invention, which may be proteinaceous, or nucleic acids encoding them, may be isolated and/or purified from the C42 bacteria described above, in substantially pure or homogeneous form, or free or substantially free of other materials from the bacterial strain of origin. Where used herein, the term "isolated" encompasses all of these possibilities. Equally, the C42 agent may be wholly or partially synthetic. In particular they may be 'recombinantly' produced from nucleic acid sequences which are not found together in nature (do not run contiguously) but which have been ligated or otherwise combined artificially.

Methods of purifying proteins from heterogenous mixtures are well known in the art (eg. selective precipitation, proteolysis, ultrafiltration with known molecular weight cut-off filters, ion-exchange chromatography, gel filtration,

etc.). A particularly useful initial technique in this regard is ultracentrifugation. Further methods which are known to be suitable for protein purification are disclosed in "Methods in Enzymology Vol 182 - Guide to Protein Purification" Ed. M P Deutscher, Pub. Academic Press Inc. Nematocidal activity may be assessed using a spread assay as discussed below.

Nucleic acids & variants

In one aspect of the present invention there is provided a nucleic acid molecule encoding a toxin of the present invention, as described above.

In the light of the present disclosure, nucleic acids may be isolated using PCR or southern blotting or other techniques well known to those skilled in the art. This requires the use of two primers to specifically amplify target nucleic acid, so preferably two nucleic acid molecules with sequences characteristic of the C42 toxin isolated as above are employed. Using RACE PCR, only one such primer may be needed (see "PCR protocols; A Guide to Methods and Applications", Eds. Innis et al, Academic Press, New York, (1990)).

In a further aspect of the present invention there are disclosed nucleic acids which are variants of the C42 toxin. A variant nucleic acid molecule shares homology (or identity) with all or part of the C42 sequences discussed above.

Preferably sequence comparisons are made using FASTA and FASTP (see Pearson & Lipman, 1988. Methods in Enzymology 183: 63-98). Parameters are set, using the default matrix

blosum62, as follows:
Gapopen (penalty for the first residue in a gap): -12 for proteins / -16 for DNA
Gapext (penalty for additional residues in a gap): -2 for proteins / -4 for DNA
KTUP word length: 2 for proteins / 6 for DNA.

Homology may be at the nucleotide sequence and/or encoded amino acid sequence level. Preferably, the nucleic acid and/or amino acid sequence shares at least about 70, 80, or 85% homology, most preferably at least about 90%, 95%, 96%, 97%, 98% or 99% homology.

Another method for assessing homology at the nucleic acid level is by hybridization screening. One common formula for calculating the stringency conditions required to achieve hybridisation between nucleic acid molecules of a specified sequence homology is shown in *Molecular Cloning: a Laboratory Manual:* 2nd edition, Sambrook *et al,* 1989, Cold Spring Harbor Laboratory Press:

T_m = 81.5°C + 16.6Log [Na+] + 0.41 (% G+C) - 0.63 (% formamide) - 600/#bp in duplex

Variants of the present invention can be artificial nucleic acids. Alternatively they may be novel, naturally occurring, nucleic acids, isolatable using the information disclosed herein. Thus a variant may be a distinctive part or fragment (however produced) corresponding to a portion of the C42 toxin. The fragments may encode particular functional parts of the agent or they may be used for probing for, or amplifying, sequences corresponding to C42 toxin. Sequence

variants which occur naturally may include homologs of the C42 toxin from other bacteria, including nematode-symbionts. Artificial variants (derivatives) may be prepared by those skilled in the art, for instance by site directed or random mutagenesis, or by direct synthesis. Preferably the variant nucleic acid is generated either directly or indirectly from an original nucleic acid encoding the C42 toxin.

Changes may be desirable for a number of reasons, including introducing or removing the following features. Sites which are required for pre- or post- translation modification. Leader or other targeting sequences (e.g. membrane or golgi locating sequences) may be added to the expressed protein to determine its location following expression. All of these may assist in efficiently cloning and expressing an active polypeptide in recombinant form. Other desirable mutation may be random or site directed mutagenesis in order to alter the activity (e.g. host specificity) or stability of the encoded polypeptide. Changes may be by way of conservative variation, i.e. substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as arginine for lysine, glutamic for aspartic acid, or glutamine for asparagine. Also included are active (nematocidal) variants having non-conservative substitutions.

The term 'variant' nucleic acid as used herein encompasses all of these possibilities. When used in the context of polypeptides or proteins it indicates the encoded expression product of the variant nucleic acid i.e. variants of C42 toxin.

Vectors & production of host cells

In one aspect of the present invention, the nucleic acid encoding the nematocidal agent(s) described above is provided in the form of a recombinant and preferably replicable vector.

Generally speaking, those skilled in the art are well able to construct vectors and design protocols for recombinant gene expression. Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. For further details see, for example, Sambrook *et al* (1989) supra.

"Vector" as used herein includes, *inter alia*, any plasmid, cosmid, phage or *Agrobacterium* binary vector in double or single stranded linear or circular form which may or may not be self transmissible or mobilizable, and which can transform prokaryotic or eukaryotic host either by integration into the cellular genome or exist extrachromosomally (e.g. autonomous replicating plasmid with an origin of replication).

Preferably the nucleic acid in the vector is under the control of, and operably linked to, an appropriate (optionally inducible) promoter or other regulatory elements for transcription in a host cell such as a microbial, e.g. bacterial, yeast, filamentous fungal or plant cell. The vector may be a bi-functional expression vector which functions in multiple hosts. In the case of genomic DNA, this may contain its own promoter or other regulatory elements and

in the case of cDNA this may be under the control of an appropriate promoter or other regulatory elements for expression in the host cell.

The agent may be used as part of a viral vector which is itself pathogenic to nematodes.

Also of interest in the present context are nucleic acid constructs which operate as plant vectors. Specific procedures and vectors previously used with wide success upon plants are described by Guerineau and Mullineaux (1993) (Plant transformation and expression vectors. In: Plant Molecular Biology Labfax (Croy RRD ed) Oxford, BIOS Scientific Publishers, pp 121-148). Suitable vectors may include plant viral-derived vectors (see e.g. EP-A-194809). Suitable promoters which operate in plants include the Cauliflower Mosaic Virus 35S (CaMV 35S). Other examples are disclosed at pg 120 of Lindsey & Jones (1989) "Plant Biotechnology in Agriculture" Pub. OU Press, Milton Keynes, UK.

Host cells

The toxin genes or gene fragments encoding the nematocidal agents of the subject invention may be introduced into a host cell (microbial, animal or plant). Expression of the toxin gene in the host cell results, directly or indirectly, in the intracellular production and maintenance of the nematocide.

Thus the present invention also provides methods comprising introduction of such a construct into a plant cell or a microbial cell and/or induction of expression of a construct

within a cell, by application of a suitable stimulus e.g. an effective exogenous inducer.

Hosts can be used simply to generate quantities of toxin which can be employed in situ in suitable treated cells, or alternatively (with suitable hosts, e.g., *Pseudomonas*) viable microbes can be applied to the sites of nematodes where they will proliferate and where they or their products can be ingested by the nematodes. Higher organisms, preferably plants, can also be engineered with the toxin. The result in each case is a control of the nematodes.

Characteristics of interest for use as a nematocide "microcapsule" (i.e. a vehicle for the active agent) include protective qualities for the nematocide, such as thick cell walls, pigmentation, and intracellular packaging or formation of inclusion bodies; leaf affinity; lack of mammalian toxicity; attractiveness to nematodes for ingestion; ease of killing and fixing without damage to the toxin; and the like.

Treated host cells

Where the cell is treated, the cell will usually be intact and be substantially proliferative form when treated, rather than in a spore form, although in some instances spores may be employed. Treatment of the microbial cell, e.g. a microbe containing the bacterial toxin gene or gene fragment, can be by chemical or physical means, or by a combination of chemical and/or physical means, so long as the technique does not deleteriously affect the properties of the toxin, nor diminish the cellular capability in protecting the toxin.

Viable hosts

Where the toxin gene or gene fragment is introduced via a suitable vector into a microbial host, and said host is applied to the environment in a living state, it is preferable that microorganism hosts are selected which are known to occupy the "phytosphere" (phylloplane, phyllosphere, rhizosphere, and/or rhizoplane) of one or more crops of interest. These microorganisms are selected so as to be capable of successfully competing in the particular environment (crop and other insect habitats) with the wild-type microorganisms, provide for stable maintenance and expression of the gene expressing the polypeptide pesticide, and, desirably, provide for improved protection of the nematocide from environmental degradation and inactivation.

A large number of microorganisms are known to inhabit the phylloplane (the surface of the plant leaves) and/or the rhizosphere (the soil surrounding plant roots) of a wide variety of important crops. These microorganisms include bacteria, algae, and fungi. Of particular interest are microorganisms, such as bacteria, e.g., genera *Pseudomonas, Erwinia, Serratia, klebsiella, Xanthomonas, Streptomyces, Rhizobium, Rhodopseudomonas, Methylophilius, Agrobacterium, Acetobacter, Lactobacillus, Arthrobacter, Azotobacter, Leuconosroc,* and *Alcaligenes;* fungi, particularly yeast, e.g., genera *Saccharomyces, Cryprococcus, Kluyveromyces, Sporobolomyces, Rhodororula, and Aureobasidium.*

Plants as hosts

Nucleic acid encoding the nematocides of the present

invention can be introduced into plant cells using any suitable technology, such as a disarmed Ti-plasmid vector carried by *Agrobacterium* exploiting its natural gene transfer ability (EP-A-270355, EP-A-0116718, NAR 12(22) 8711 - 87215 1984), particle or microprojectile bombardment (US 5100792, EP-A-444882, EP-A-434616) microinjection (WO 92/09696, WO 94/00583, EP 331083, EP 175966, Green *et al.* (1987) *Plant Tissue and Cell Culture*, Academic Press), electroporation (EP 290395, WO 8706614 Gelvin Debeyser) other forms of direct DNA uptake (DE 4005152, WO 9012096, US 4684611), liposome mediated DNA uptake (e.g. Freeman *et al. Plant Cell Physiol.* 29: 1353 (1984)), or the vortexing method (e.g. Kindle, *PNAS U.S.A.* 87: 1228 (1990d) Physical methods for the transformation of plant cells are reviewed in Oard, 1991, *Biotech. Adv.* 9: 1-11.

Agrobacterium transformation is widely used by those skilled in the art to transform dicotyledonous species. It has also been used with filamentous fungi (see de Groot et al, 1998, Nature Biotechnology 16: 839-842).

Recently, there has also been substantial progress towards the routine production of stable, fertile transgenic plants in almost all economically relevant monocot plants (see e.g. Hiei *et al.* (1994) *The Plant Journal* 6, 271-282)). Microprojectile bombardment, electroporation and direct DNA uptake are preferred where *Agrobacterium* alone is inefficient or ineffective. Alternatively, a combination of different techniques may be employed to enhance the efficiency of the transformation process, eg bombardment with *Agrobacterium* coated microparticles (EP-A-486234) or microprojectile bombardment to induce wounding followed by co-cultivation

with *Agrobacterium* (EP-A-486233).

Generally speaking, following transformation, a plant may be regenerated, e.g. from single cells, callus tissue or leaf discs, as is standard in the art. Almost any plant can be entirely regenerated from cells, tissues and organs of the plant. Available techniques are reviewed in Vasil *et al.*, *Cell Culture and Somatic Cell Genetics of Plants, Vol I, II and III, Laboratory Procedures and Their Applications*, Academic Press, 1984, and Weissbach and Weissbach, *Methods for Plant Molecular Biology*, Academic Press, 1989.

The generation of fertile transgenic plants has been achieved in the cereals rice, maize, wheat, oat, and barley (reviewed in Shimamoto, K. (1994) *Current Opinion in Biotechnology* 5, 158-162; Vasil, *et al.* (1992) *Bio/Technology* 10, 667-674; Vain et al., 1995, *Biotechnology Advances* 13 (4): 653-671; Vasil, 1996, *Nature Biotechnology* 14 page 702).

Combination nematocides

In further embodiments of the invention, bacteria associated with entomopathogenic nematodes or the toxins discussed above are used in conjunction with nematocidal *B thuringiensis* strains (e.g. from WO 92/19739) or pesticidal materials derived therefrom.

Materials for use in the present invention

The present invention also embraces materials for use in the methods above. These materials include the novel bacterial strains which are associated symbiotically with an

entomopathogenic nematode and which are capable of controlling a target nematode. In particular the invention encompasses strain C42 in isolated or substantially isolated form, or strains having the characteristics of C42 (including nematocidal activity assessed as below).

Also embraced are compositions and formulations of these bacteria. These may comprise or consist of wettable powders, granules or dusts, mixed with various inert materials, such as inorganic minerals (phyllosilicates, carbonates, sulfates, phosphates, methylcellulose, xanthan gum and the like) or botanical materials (powdered corncobs, rice hulls, walnut shells, peat moss, vermiculite, soil, seeds, other plant tissue and the like). The formulations may include spreader-sticker adjutants, stabilizing agents or surfactants. Liquid formulations may be aqueous-based or non-aqueous and employed as foams, gels, suspensions, emulsifiable concentrates, or the like. The ingredients may include rheological agents, surfactants, emulsifiers, dispersants, or polymers.

Bacteria may be mixed with other material while in freeze-dried form, encapsulated in biodegradable or water-soluble material, or otherwise treated to prolong their viability or decrease their levels of metabolic activity during handling. If desired, the carrier material may contain assimilatable nutrient sources to support proliferation of the bacteria.

Also included are purified or substantially purified nematocidal agents (particularly proteinaceous agents) isolated from the strains discussed above.

Thus the invention further discloses nematocidal compositions

comprising one or more agents as described above. Such compositions preferably further comprise other nematocidal materials from other *Xenorhabdus* species or non-*Xenorhabdus* species. These other materials may be chosen such as to have complementary properties to the agents described above, or act synergistically with it.

Toxins of the invention for use with animals can be adapted to be administered orally in a unit dosage form such as a capsule, bolus or tablet, or as a liquid drench when used as an anthelmintic in mammals, and in the soil to control plant nematodes. The drench is normally a solution, suspension or dispersion of the active ingredient, usually in water, together with a suspending agent such as bentonite and a wetting agent or like excipient. Generally, the drenches also contain an antifoaming agent. Drench formulations generally contain from about 0.001 to 0.5% by weight of the active compound. Preferred drench formulations may contain from 0.01 to 0.1% by weight, the capsules and boluses comprise the active ingredient admixed with a carrier vehicle such as starch, talc, magnesium stearate, or dicalcium phosphate. Where it is desired to administer the toxin compounds in a dry, solid unit dosage form, capsules, boluses or tablets containing the desired amount of active compound usually are employed. These dosage forms are prepared by intimately and uniformly mixing the active ingredient with suitable finely divided diluents, fillers, disintegrating agents andior binders such as starch, lactose, talc, magnesium stearate, vegetable gums and the like. Such Unit dosage formulations may be varied widely with respect to their total weight and content of the antiparasitic agent, depending upon the factors such as the type of host animal to be treated, the

severity and type of infection and the weight of the host.

When the active compound is to be administered via an animal feedstuff, it is intimately dispersed in the feed or used as a top dressing or in the form of pellets which may then be added to the finished feed or, optionally, fed separately. Preferably, a carrier for feed administration is one that is, or may be, an ingredient of the animal ration. Suitable compositions include feed premixes or supplements in which the active ingredient is present in relatively large amounts and which are suitable for direct feeding to the animal or for addition to the feed either directly or after an intermediate dilution or blending step. Typical carriers or diluents suitable for such compositions include, for example, distillers' dried grains, corn meal, citrus meal, fermentation residues, ground oyster shells, wheat shorts, molasses solubles, corn cob meal, edible bean mill feed, soya grits, crushed limestone and the like.

Alternatively, the antiparasitic compounds may be administered to animals parenterally, for example, by intraluminal, intramuscular, intratracheal, or subcutaneous injection, in which event the active ingredient is dissolved or dispersed in a liquid carrier vehicle. For parenteral administration, the active material is suitably admixed with an acceptable vehicle, preferably of the vegetable oil variety, such as peanut oil, cotton seed oil and the like. Other parenteral vehicles, such as organic preparations using solketal, glycerol, formal and aqueous parenteral formulations, are also used. The active compound or compounds are dissolved or suspended in the parenteral formulation for administration; such formulations generally contain from

0.005 to 5% by weight of the active compound.

Further aspects of the invention include nucleic acids, vectors and host cells containing a heterologous construct according to the present invention, especially a plant or a microbial cell.

Such microbial cells may be treated as described in the methods above. Examples of chemical reagents are halogenating agents. Other suitable techniques include treatment with aldehydes, such as formaldehyde and glutaraldehyde; anti-invectives, such as zephiran chloride and cetylpyridinium chloride; alcohols, such as isopropyl and ethanol; various histologic fixatives, such as Bouin's fixative and Helly's fixative (See: Humason, Gretchen L., Animal Tissue Techniques, W.H. Freeman and Company, 1967); or a combination of physical (heat) and chemical agents that preserve and prolong the activity of the toxin produced in the cell when the cell is administered to the host animal. The method of inactivation or killing retains at least a substantial portion of the bio-availability or bioactivity of the toxin.

In all of the compositions discussed above, the nematocide concentration may vary widely depending upon the nature of the particular formulation, particularly whether it is a concentrate or to be used directly. The nematocide will be present in at least 1% by weight and may be 100% by weight. The dry formulations will have from about 1-95% by weight of the nematocide while the liquid formulations will generally be from about 16% by weight of the solids in the liquid phase. The formulations will generally have from about 10^2 to about 10^{10} cells/mg, more preferably 10^7 to about 10^9 cells/mg.

These formulations will be administered at about 50 mg (liquid or dry) to 1 kg or more per hectare. The formulations can be applied to the environment of the nematodes, e.g., plants, soil or water, by spraying, dusting, sprinkling, or the like.

In addition to the above the invention includes plant cells which have been transformed with the toxin genes of the present invention, and plants which include such plant cells.

The invention will now be further described with reference to the following non-limiting Figures and Examples. Other embodiments of the invention will occur to those skilled in the art in the light of these.

EXAMPLES

Example 1 - identification of strain C42

Strain C42 was obtained using an insect entrapment method. Insects which were killed on the surface of a soil sample were observed under a microscope at high magnification. Any that contained high numbers of bacteria and not fungal hyphae were presumed to have been killed by insect parasitic nematodes. The identified presence of nematodes also aids this identification step, but it is not essential. These samples were plated on to NBTA media (see Poinar & Thomas, 1984 Nematodes p238-280 in "Laboratory guide to insect pathogens amd parasites" Eds. Poiner & Thomas, Pub. Plenum Press, New York). Any colonies that developed that had characteristic features (e.g. morphology, size, colour) of *Xenorhabdus* or *Photorhabdus* strains were selected. Non-

luminescent colonies were presumptively identified as *Xenorhabdus*. The identity of those having nematocidal activity as assessed in Example 2, is further confirmed using 16s rRNA sequence data (see Brunel et al 1997, Applied and Environmental Microbiology 63,2: 574-580).

Example 2 - Cell growth and preservation

Subcultures of the *Xenorhabdus* species C42 were used to inoculate three, 9 cm diameter petri dishes containing L agar (10g tryptone, 5g Yeast Extract, 5g NaCl and 15g agar per Lt). Plates were incubated for 48hrs at 26°C and the resulting growth harvested by scraping off bacterial cells and thoroughly resuspending in 40mls of 5% w/v lactose. The cells were washed once by centrifugation (5000g for 10 mins), resuspended in 10 mls of 5% lactose, dispensed into 1ml aliquots and freeze dried (-60°C for 48hrs) for long term storage at 2°C C. Other stocks were re-suspended in nutrient broth containing 10% w/v glycerol (Protect) and frozen at -70°C.

Example 3- Activity of cells against *Caenorhabditis elegans*

The bioassays were performed by allowing *C. elegans* to feed on live bacterial cell suspensions spread over the surface of Luria broth agar (Luria broth containing 1.2%w/v agar) in segmented square petri dishes (2.5 x 2.5cm per test well). A minimum of three test wells, each containing 50-100 nematodes were used for each test. Mortalities were recorded after 3 days at 18°C.

C. elegans was cultured on *Escherichia coli* at 18°C on 9cm

diam LB agar plates. Once the nematodes had colonised the complete plate they were re-subbed on a fresh plate to maintain stocks and the remainder re-suspended in 40ml LB. The tube was allowed to stand for 15 min and the nematodes settled to the bottom. The concentrated nematodes were removed using a sterile pipette and placed in 40 mls of fresh LB. The process was repeated 5 more times to wash the nematodes away from the *E. coli* cells. The nematodes were then diluted so that approx. 50 nematodes were present in 50 µl of LB.

The *Xenorhabdus* cells used, were cultured in LB at 30°C / 100 rpm for 24 hours and 50 µl spread on to the surface of each test well. The control *E. coli* cells were treated in a similar way but incubated at 37°C for growth. After application the wells were air dried for 30 min, and 50 µl of the nematode suspension placed in each well. Again the wells were air dried for 30 min. Plates were incubated at 18°C with 80% relative humidity for 3 days.

Xenorhabdus spp C42 gave 100% mortality, as compared with 0% for certain other *Xenorhabdus* bacterial strains and *E. coli*. Thus these results clearly show that cells from *Xenorhabdus* C42 are an effective nematocide.

Example 4- Field trials

Activity of strains selected in accordance with the above methods, or from depositary institutions which include bacteria which in nature are associated symbiotically with entomopathogenic nematodes, may be further assessed in field trials as follows.

Symbiotic bacteria, which are believed to be viable in the soil even on the absence of their nematode host, can be inoculated into one or more portions of a field which is infested with nematodes, or into containers containing unsterilised soil from such a field. The bacteria can be inoculated onto the roots of plants, or into seeds. Periodically treated and untreated areas or containers can be assayed for nematode larva, egg, or cyst counts and for the presence of the inoculated bacteria by methods well known to those skilled in the art. A reduction in the number of nematode counts in areas in which the symbiote bacteria are present indicates control of the nematodes otherwise found in the untreated areas or samples.